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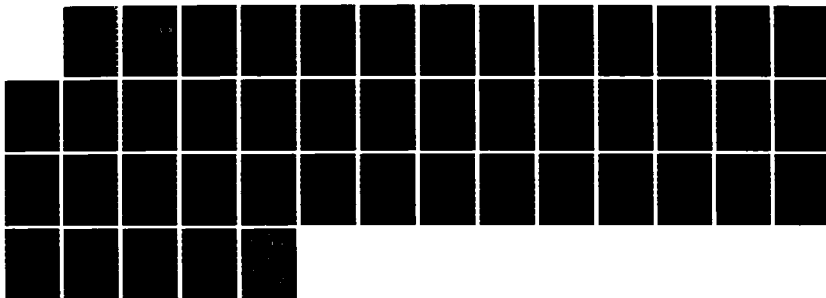
PATHOGENESIS OF CELL INJURY BY RICKETTSIA CONORII(U)
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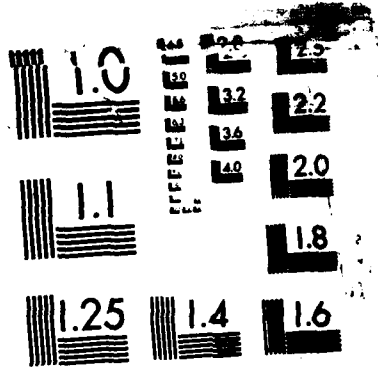
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Report Number 1

Pathogenesis of Cell Injury by
Rickettsia conorii

Annual Summary Report

David H. Walker, M.D.

June 15, 1984

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U.S. Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland 21701

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University of North Carolina at Chapel Hill
Chapel Hill, North Carolina 27514

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tissues were investigated by light microscopy, histochemistry, immunofluorescence, and electron microscopy. In vitro models of cell injury by R. conorii included parabiologic chambers and the plaque model.

Biopsies of 17 taches noires and 4 liver biopsies from patients with boutonneuse fever and necropsy tissues from two fatal cases of South African tick bite fever and amputated, gangrenous fingers from another patient with severe Rickettsia conorii infection were examined. R. conorii were identified in 11 taches noires, none of the liver biopsies, the endothelium or macrophages of the kidneys, brain, meninges, liver, spleen, heart, lung, lymph node, skin, and pancreas of the autopsy cases, and the blood vessels of the partially necrotic zone between the viable tissue and the mummified, necrotic tissue of the amputated fingers. Significant vascular injury and infiltrating small lymphocytes and large mononuclear cells were observed in most of these locations. The lesions were similar to other rickettsioses such as typhus fever and Rocky Mountain spotted fever. Vasculitis was more prominent than vascular thrombosis.

In vitro studies employing parabiologic chambers and the plaque model yielded no evidence for an exotoxin or soluble enzyme in cell injury by R. conorii, but did document that cell injury could be blocked by inhibitors of phospholipase A₂ or trypsin-like protease.

These results lead us to conclude that a rapid diagnostic test has been achieved, that visceral lesions and rickettsial distribution occur in fatal cases, and that further studies of taches noires and protease and phospholipase inhibitors are important.

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Summary

This work was undertaken to determine the pathogenic mechanism by which Rickettsia conorii causes disease. R. conorii, an organism that has been neglected in spite of its widespread distribution and pathogenic qualities, was studied in human subjects and in vitro. The purpose of the work is to elucidate the pathology of boutonuse fever and the pathogenic mechanisms which might be blocked therapeutically or prophylactically. Human tissues were investigated by light microscopy, histochemistry, immunofluorescence, and electron microscopy. In vitro models of cell injury by R. conorii included parabiotic chambers and the plaque model.

Biopsies of 17 taches noires and 4 liver biopsies from patients with boutonuse fever and necropsy tissues from two fatal cases of South African tick bite fever and amputated, gangrenous fingers from another patient with severe Rickettsia conorii infection were examined. R. conorii were identified in 11 taches noires, none of the liver biopsies, the endothelium or macrophages of the kidneys, brain, meninges, liver, spleen, heart, lung, lymph node, skin, and pancreas of the autopsy cases, and the blood vessels of the partially necrotic zone between the viable tissue and the mummified, necrotic tissue of the amputated fingers. Significant vascular injury and infiltrating small lymphocytes and large mononuclear cells were observed in most of these locations. The lesions were similar to other rickettsioses such as typhus fever and Rocky Mountain spotted fever. Vasculitis was more prominent than vascular thrombosis.

In vitro studies employing parabiotic chambers and the plaque model yielded no evidence for an exotoxin or soluble enzyme in cell injury by R. conorii, but did document that cell injury could be blocked by inhibitors of phospholipase A2 or trypsin-like protease.

These results lead us to conclude that a rapid diagnostic test has been achieved, that visceral lesions and rickettsial distribution occur in fatal cases, and that further studies of taches noires and protease and phospholipase inhibitors are important.

Foreword

For the protection of human subjects, the investigator has adhered to policies of applicable Federal Law 45CFR46.

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Statement of Problem

Spotted fever group rickettsiae including Rickettsia conorii, R. sibirica, and R. akari are important potential causes of military health problems. In order to meet the challenges of these diseases to the health of groups of soldiers who enter zoonotic areas, methods of effective prevention, improved diagnosis, and optimal treatment are required. Development of an effective vaccine offers the best hope for prevention of boutonneuse fever and other spotted fever group rickettsioses. No effective vaccine exists for any of these rickettsial diseases. Because most effective vaccines for prokaryotic organisms rely upon interdiction of the specific pathogenic mechanism of the organism, e.g., diphtheria and tetanus, it is important to elucidate the pathogenic mechanism of cell injury by R. conorii. The failure of killed rickettsial and bacterial vaccines, e.g., Rocky Mountain spotted fever, typhoid fever, and cholera, may be a result of a lack of stimulation of the immune system to block crucial pathogenic steps. The goal of this research contract is to determine the pathogenic mechanism for R. conorii. Laboratory research on hypothetical rickettsial pathogenic effects must be compared with observations on the human disease in order to assure as well as possible the relevance and reality of working models of the host-parasite interaction. The problems of lack of information on the pathology of boutonneuse fever, the human ultrastructural lesions for any rickettsiosis, and the composition of the immune and inflammatory cell populations actually present in foci of rickettsial infection in humans are addressed in this research project. Diagnosis of boutonneuse fever, North Asian tick typhus, and rickettsialpox is an unsure affair with a considerable amount of room for error. Misdiagnosis and delayed diagnosis result in prolonged illness, need for more care often including nursing and hospitalization, and failure to institute epidemiologic preventive measures. Diagnosis is a clinical one in the acute stage of the illness. Yet, clinical features are variable and do not always lead to a timely correct diagnosis. There has been no rapid, acute laboratory diagnostic method. Serologic diagnosis is a retrospective tool employed during convalescence or in the late stage of the illness. There are few facilities in the world for isolation of R. conorii, and the laboratory procedure for isolation is both cumbersome and long. A diagnostic test that can be applied during the acute stage of illness is an expected spinoff of this research project.

Background

Rickettsial diseases occur over a wide geographic distribution, are firmly entrenched ecologically, and pose an important threat to both military and public health.

Members of the genus Rickettsia are classified into three groups on the basis of shared group antigens: spotted fever group, typhus group, and scrub typhus group. All are obligate intracellular bacteria which spend at least a portion of their life in arthropod hosts such as ticks, mites, fleas, or lice. They all affect man in a similar fashion with hematogenous spread and

infection of vascular endothelium producing increased vascular permeability and vasculitis in multiple organ systems. These rickettsiae include the etiologic agents of diseases that have been documented as major military health problems. Rickettsia prowazekii has affected the outcome of numerous military campaigns for centuries. R. tsutsugamushi was a severe problem in Asia and the western Pacific theaters during World War II and infected soldiers in the Viet Nam War. These rickettsiae have continued to attract research support. Although R. conorii has received far less attention, it too has been documented as an important cause of illness among troops in South Africa. R. conorii is a member of the spotted fever group of rickettsiae along with other human pathogens including R. rickettsii (Rocky Mountain spotted fever), R. akari (rickettsialpox), R. sibirica (North Asian tick typhus), and R. australis (Queensland tick typhus). Isolates of spotted fever group rickettsiae from the Mediterranean basin, where the disease is known as boutonneuse fever, East Africa (Kenya tick typhus), South Africa (South African tick typhus), and the Indian subcontinent (India tick typhus), were all shown to be members of the same species, R. conorii, by the mouse toxin neutralization test. Data presented by Myers and Wisseman on DNA hybridizations among the spotted fever group rickettsiae have documented close relationships among various strains of R. conorii including rickettsiae associated with the severe disease occurring in Israel and R. rickettsii. Many of these hybridizations were in the range of 90-100% homology.

Infection of man with various strains of R. conorii occurs in a widespread geographic distribution in the Old World with well-documented disease in the Mediterranean basin, Africa, and the Middle East from Israel to India. In the Mediterranean basin, the disease is endemic in Portugal, Spain, southern France, Italy, Greece, Romania, Turkey, Morocco, Algeria, Tunisia, Libya, and Egypt as well as in the margins of the Black Sea and the Caspian basin. More recently it has been reported from South Africa, Kenya, India, Pakistan, Togo, Ethiopia, Cameroun, and Israel.

In the majority of the areas where the disease is endemic, it occurs as sporadic cases during the summer months with little variation in the annual numbers of cases reported. Scafidi notes that there were 107 cases in Israel in 1974, around 30 annual cases in Tunisia from 1961-1975, and 20 annual cases in Marseille from 1925-1930. He and Bourgeade et al, however, point out that these numbers do not reflect the reality since the great majority of patients are treated at home and are not reported. This is also an explanation for the scarcity of information about the prevalence of the disease.

The low endemicity that prevails in the majority of the affected areas has changed significantly in Italy where, since 1975, there was a sharp increase in the incidence of the disease. Indeed, from an average of less than 10 cases per year up to 1972 the number of cases in Sicily increased progressively to reach 219 cases in 1979. Similar increases were observed in other regions of Italy as Liguria, Sardinia, and Lazzio; in this last mentioned region that includes the city of Rome, there were 369 cases reported in 1979. Besides Rome, the disease has also been reported in suburban and urban Marseille, and there are data that it is also increasing in Spain and Portugal.

The causes for such a rapid increase in the incidence of boutonuse fever in Italy are not apparent. The Italians have suggested several possible explanations: 1) increase in the number of vectors, 2) introduction of new vectors, and 3) changes in the ecosystem. There have been some very interesting observations on the isle of Ustica where, after the recent introduction of wild rabbits, there was an explosive proliferation of Hyalomma excavatum, a tick that had rarely been found in the island previously. Gilot, et al also mention the possibility of adaptation of certain species of ticks, parasites of wild animals, to human dwellings and the potential consequences of the transmission of boutonuse fever.

What is happening in Italy may occur in other regions. Weyer, reviewing the subject of rickettsioses in 1978, said "Despite the great successes in control, none of the rickettsioses pathogenic for man have been eradicated. Therefore, it is necessary to preserve the knowledge about these once devastating and important diseases because the present situation could change suddenly."

Indeed, recent data have demonstrated that several different species of ticks harbor R. conorii not only in the known endemic areas but also in regions where the human disease is not recognized including Pakistan, Armenia, Thailand, areas of France, Czechoslovakia, Austria, and Germany.

Boutonuse fever is transmitted to man from ticks, most frequently by Rhipicephalus sanguineus. Infected ticks transmit the disease through their infected salivary secretions during the bite; exceptionally the agents may invade the human host from infectious tick material through abrasions in the skin or through the conjunctivae. There are references that report the disease being acquired by persons who rubbed their eyes after deticking dogs. The agent appears innocuous to the tick which also serves as reservoir for R. conorii which is transmitted transovarially in ticks. Small wild mammals are the source of blood meals for immature forms of R. sanguineus. Dogs, and on occasion man, are the source of blood meals for the adult stage. The following species of ticks, besides the common vector Rhipicephalus sanguineus, have been reported to harbor R. conorii: Ixodes ricinus, R. hexagonus, Dermacentor marginatus, and D. reticulatus in France; Haemaphysalis leachii, Amblyomma hebraeum, Rhipicephalus appendiculatus, R. evertsi, and Hyalomma marginatus rufipes in South Africa; Amblyomma variegatum and Hyalomma albiparvum in Kenya; Ixodes granulatus in Malaysia; Rhipicephalus simus, Amblyomma variegatum, A. cohaerens, and A. gemma in Ethiopia; and Rhipicephalus bursa, Hyalomma marginatum, H. lusitanicum, and Haemaphysalis punctata in Sicily. Moreover, serological tests in wild and domestic animals have shown that antibodies against R. conorii are present in several species in many regions, some of them far away from the known endemic areas. In Sicily, 20% of dogs harbor R. sanguineus and 29-71% of them have antibodies to R. conorii identified by immunofluorescence test. Serologic tests have identified antibodies against R. conorii in large numbers of healthy persons: in Africa, 13% of sera contained antibodies in an investigation in Cameroun and similar results were reported from Niger, Zaire and the Central African republic; in Greece 16% of 560 sera from healthy persons were positive; data from France indicate that positive serology in healthy persons has

been observed in Caen, Nantes, and Lyon. In one endemic area of Sicily 19.3% of healthy subjects had positive immunofluorescence assay for anti- R. conorii antibodies. Not all of these studies employed the same serological tests, and there is variation in specificity among different tests. Some, however, used specific immunofluorescence techniques.

All the data above presented confirm the suggestion of Weyer that the stage is set for an increase in the frequency of boutonneuse fever and that this may occur in many different areas of the world.

Recently there have been reports of cases of boutonneuse fever in German and Swiss tourists who had returned from endemic areas and even of cases in American tourists returning from Africa. Interestingly, a tick was found on one of these patients that might, if circumstances had been favorable, have become established in an American ecological niche. Cases have also been reported in persons living in Paris.

Human illness caused by R. conorii infection is usually an incapacitating febrile exanthem. Although death is rare, some strains of R. conorii possess the capability of producing severe disease requiring hospitalization and critical medical and nursing care. The disease usually resolves spontaneously in one or two weeks, this period being reduced by appropriate antibiotic therapy which may be given at home. It is necessary to emphasize that even being mild it is incapacitating and in a minority of cases can be severe or even fatal; moreover, in certain regions, as apparently is the case in Israel, it can assume a more severe course similar to the picture of Rocky Mountain spotted fever. The minority of patients seeking hospital care apparently do not manifest any special features or increased severity of their illness. Men are slightly more frequently affected than females and the disease occurs at all ages being, however, uncommon in the very young and very old. Most of the patients report contact with dogs, ticks, or recent visit to endemic areas; others are farmers or hunters. The incubation period varies from 7 to 14 days, but can be as short as 4 or as long as 22 days. In the majority of the cases the patient remembers being bitten by a tick and from 33% to 92% of them have an eschar (tache noire) at the site of the tick bite. Less frequently they have acute unilateral conjunctivitis.

The disease is initiated by a sudden increase in temperature to levels as high as 40°C; at the same time the patients complain of joint and muscle pain and violent, persistent headache that is frequently retroorbital. There is also congestion of the conjunctivae and mild lymphadenopathy. These manifestations coincide with the appearance of the eschar. Four to 5 days after the beginning of the fever the typical rash appears; it is first observed on the limbs but rapidly expands to trunk and face with palms and soles being also involved. In some cases even the oral mucosa presents an exanthem. In the beginning the rash appears as erythematous macules that rapidly change to a maculopapular pattern and eventually become nodular or button-like, as the name describes. The early lesions are light pink, but some of the older ones may become darker or hemorrhagic. The rash occurs in successive bouts so that lesions in different phases may be observed side by side.

Fever persists for 7-14 days and during this period 46% of

the patients develop splenomegaly, 20% hepatomegaly, and some patients, signs of pulmonary congestion. Diarrhea, constipation and vomiting may also occur. Neurological signs of meningeal irritation as nuchal rigidity or Kernig's sign as well as obtundation and even coma can be observed in a minority of the cases. These more severe manifestations occur mainly in older or debilitated persons; they are exceptional in children. Recovery is uneventful without any sequelae. Mortality is low, less than 1%. In a few cases, however, complications occur; they are rare and, as stated, tend to occur in older debilitated persons. Scafidi *et al* describe cases of hypertoxic, "dermatotifosa" and hemorrhagic disease, the last form being associated with severe gastrointestinal or genital bleeding. They go on to describe cases with atrial fibrillation, myocardial ischemia, and renal complications. A series of French publications describe "atypical rickettsiosis" with pericarditis, pleuritis, and pneumonitis. Some of the cases, however, did not present with eschars and the final diagnosis was made by positive microagglutination tests according to the method of Giroud, thus raising doubts concerning the diagnosis. In Israel, however, there have been some very interesting cases of tick-borne rickettsiosis with severe renal insufficiency requiring dialysis; in these cases, there are questions about the exact classification of the etiologic agent that did not conform exactly with the antigenic structure of *R. conorii*. More recently severe and fatal cases have been described in South Africa and France.

The clinical feature that is most significant diagnostically in *R. conorii* infection is the tache no're which develops at the site of tickbite in approximately 50% of cases. The tache noire, or black spot, is a zone of dermal and epidermal necrosis which generally appears prior to onset of fever and rash. Conor and Burch did not describe eschars in the original description of human *R. conorii* infection in 1910. Tache noire is a French term and was introduced in 1925 by Pieri to refer to the tickbite site eschar in boutonneuse fever. Thereafter, the term tache noire seems to have been used continuously. Similar eschars are frequently observed in scrub typhus (*R. tsutsugamushi*), North Asian tick typhus (*R. sibirica*), rickettsialpox, (*R. akari*), and Queensland tick typhus (*R. australis*). Eschars are rarely observed in Rocky Mountain spotted fever and do not occur in typhus fever and murine typhus. Thus, eschars are seen only in rickettsioses transmitted by inoculation of infected salivary secretions by ticks and mites and are not observed in rickettsioses transmitted by scratching rickettsia-containing louse or flea feces into the skin. Patients who develop boutonneuse fever after accidental introduction of infected tick constituents into the conjunctiva do not have eschars, but manifest conjunctivitis at the portal of entry.

Our laboratory has described the clinical features, brightfield microscopic pathology, and distribution of *R. rickettsii* in eschars which occurred in two fatal cases of Rocky Mountain spotted fever examined at autopsy. These eschars consisted of a 8 x 10 mm oval region of necrotic epidermis and underlying dermis. The necrotic zone was surrounded by a zone of blood vessels that were injured with extensive thrombosis and intramural and perivascular mononuclear inflammatory cells. Immunohistochemical examination revealed very large quantities of *R. rickettsii* in the endothelium and vascular wall of these blood vessels.

There is some degree of controversy about the role of constituents of tick salivary secretions such as enzymes associated with tickbite in the pathogenesis of the tache noire. Experimental studies suggest that the dose of inoculum rather than the tickbite itself is crucial. Inoculation of a large dose of R. rickettsii, a generally nonescharogenic rickettsia, into human skin by syringe and needle produces eschars. Inoculation of R. conorii into the skin of syphilitic subjects as pyrotherapy produced taches noires proportional to the quantity of rickettsiae injected. Even nonescharogenic R. mooseri produces eschars in the skin of guinea pigs injected intradermally by syringe and needle with a large dose of rickettsiae. Not all monkeys inoculated with R. tsutsugamushi develop an eschar at the injection site; some develop only papules which do not undergo epidermal necrosis and ulceration. Thus, the tache noire appears to be an accessible lesion that contains the pathogenic mechanisms of R. conorii and the immune and inflammatory mechanisms of the host that lead to healing.

Hypothetical rickettsial pathogenic mechanisms include both those that are host-mediated and rickettsia-mediated. Host-mediated mechanisms of injury which have been proposed include immunopathology, blood coagulation, and inflammation. Rickettsia-mediated mechanisms might include an exotoxin, endotoxin, enzymes that destroy host components, metabolic competition for the host's intracellular substrates, ATP parasitism, and host cell membrane injury on rickettsial penetration into and/or release from the target cell.

Experimental evidence indicates that host-mediated pathogenic mechanisms such as immunopathology, Schwartzman phenomenon-like blood coagulation, and inflammation are not the primary mechanisms of injury in infection by R. rickettsii. Localized effects of kallikrein are probably events secondary to the primary pathogenic mechanism(s). Occlusive vascular thrombosis is infrequent, and has not been demonstrated as a primary pathogenic mechanism.

Among the hypothetical rickettsia-mediated mechanisms of injury, currently no toxin of R. rickettsii has been identified, and there is evidence against the existence of a toxin as an important pathogenic mechanism. The confusion regarding this hypothesis has originated in the so-called mouse toxin phenomenon and in erroneous analogies drawn between endotoxin and rickettsiae. Mouse toxicity depends on viable, metabolically active rickettsiae and is prevented by heating (60°C for 30 minutes), exposure to dilute formalin, rickettsial starvation, ultraviolet irradiation, specific anti-rickettsial antiserum neutralization, and a beta-lipoprotein present in some normal human sera. The pathogenesis of this phenomenon may be related to the pathophysiology of the rickettsia-host cell interaction, e.g., massive rickettsial penetration of endothelium. Rickettsiae of both the typhus and spotted fever groups have been shown to contain lipopolysaccharides. However, the endotoxin activity in bioassays including the Schwartzman phenomenon and Limulus assay was considerably less than that of potent bacterial endotoxins. Moreover, study of the adrenal in fatal RMSF has not demonstrated the pathologic lesions expected of endotoxin-mediated pathogenesis. Further evidence against the hypothesis of rickettsial toxin has been demonstrated in the plaque model. Thus, the evidence for a

rickettsial toxin of pathogenic importance is quite meager.

The plaque model has been established as a useful tool for investigation of pathogenic mechanisms of cell injury by R. rickettsii. Inoculation of confluent monolayers of primary chick embryo cells derived from 12-day old specific pathogen-free, antibiotic-free, embryonated hen's eggs with a defined quantity of R. rickettsii results in a predictable course of infection and pathologic alterations in v'tro. Each infectious unit under agarose overlay produces contiguous centrifugal spread of intracellular infection and injury to the host cell monolayer. This model produces a grossly visible plaque on day 5 after inoculation when overlaid with agarose containing the supravital dye neutral red. The plaque provides a temporal and spatial cross-section of the rickettsia-host cell interaction including rickettsial penetration, proliferation and release, and host cell cytopathologic alterations and necrosis. Morphometric analysis of the plaque and surrounding infected and uninfected cells has been performed maintaining the topographic relationships of the cells as a monolayer. The results have shown the association of intensity of infection and cytopathology at the microscopic and ultrastructural levels. There is a statistically highly significant relationship between the intensity of infection as measured by the quantity of intracellular rickettsiae and the presence of cellular injury as judged by cytopathology and necrosis. This relationship is valid independently of the apparent duration of infection. That is to say, more heavily parasitized host cells are more likely to exhibit pathologic alterations, even if they are located at the margin of the plaque, than those cells which contain fewer rickettsiae and are nearer to the center of the plaque. This study also confirms the observation of Silverman and Wisseman that the typical cytopathologic change in chick embryo cells infected with R. rickettsii is distinct dilation of the cisternae of endoplasmic reticulum. This ultrastructural finding is characteristic of the response of an injured cell to the influx of water. The utilization of the technique of maintaining the topography of the monolayer intact enabled us to determine that the uninfected cells of the monolayer even within 1 mm of the intensely infected marginal zone of the plaque were normal by ultrastructural and supravital dye staining criteria even though they were exposed to the same milieu of extracellular nutritional factors, nonspecific toxic products of metabolism and substances released from injured cells, and senescence of cultured cells. Thus, the plaque model, which has a 0.5% agarose overlay that prevents rapid, distant spread of rickettsiae and yet allows for diffusion of macromolecules, demonstrated that cell injury was limited to the more heavily parasitized cells and that there was no toxic effect on uninfected cells, even those immediately adjacent. This is strong evidence that R. rickettsii does not elaborate an extracellular toxin which affects chick embryo cells. Further studies in our laboratory have extended this observation and conclusion to Vero cells which are of primate origin and to human umbilical vein endothelial cells.

Another strong indication that R. rickettsii does not produce an important toxin resulted from observations utilizing parabiotic chambers. Specially designed flasks contained coverslips with monolayers of cells with fluid overlay in separate chambers which were separated by an 0.22 μ m millipore filter. R. rickettsii

was inoculated into one chamber of several flasks; other control flasks were observed without rickettsiae in either chamber. Inoculated monolayers developed cytopathic effect associated with heavy rickettsial infection. On the other hand, the cells in the opposite chamber remained viable with the same appearance as monolayers of unmanipulated parabiologic chambers. No toxic macromolecules injured the side of the chamber which was protected from rickettsial infection by the 0.22 μ m filter. The filter offered no barrier to the free passage of molecules between the infected and uninfected chambers. Thus, in an experimental system in which rickettsiae injured infected host cells, we demonstrated no effect of putative toxin, which would have been in equal concentration in the extracellular fluid of both chambers if it were present.

Examination of the hypothesis of competition for metabolic substrates has also failed to produce evidence to support it as a pathogenic mechanism in plaque model experiments with supplemental glutamate and glutamine. Although rickettsiae are capable of generating ATP for penetration of host cells by oxidation of glutamate, exogenous ATP from the host cell is utilized for biosynthesis of proteins and lipids by rickettsiae. This energy parasitism is mediated by an efficient rickettsial ATP/ADP transport system. No experiment has yet been designed and executed to test the hypothesis of energy parasitism as a pathogenic mechanism.

Experiments reported principally by Winkler and co-workers suggest that rickettsial penetration-associated phospholipase activity injures the host cell membrane. The work of Winkler and associates on hemolysis by viable R. prowazekii has led to an understanding of the rickettsia-host cell membrane interaction which probably forms the basis of penetration and a mechanism of cell injury. Rickettsial hemolysis may be divided into two steps, adsorption and lysis. Hemolysis is inhibited by cyanide (1 mM KCN, an inhibitor of the electron transport system), low temperature (0°C), and starvation of R. prowazekii for glutamate. Ghosts of erythrocytes exposed to Amphotericin B or digitonin, compounds which bind to the cholesterol-containing receptor sites in the erythrocytic membrane, are no longer able to adsorb rickettsiae. Adsorption and hemolysis are inhibited by adenine nucleotides, ADP, ATP, arsenite, which is a Krebs cycle inhibitor, and 2,4-dinitrophenol and m-chloro-phenylhydrazine, which are oxidative phosphorylation uncouplers. When rickettsiae are unable to generate ATP by metabolism of glutamate because of cyanide or arsenite inhibition, added ATP restores hemolytic activity of the rickettsiae. ATP, however, does not restore hemolytic activity inhibited by uncouplers. Fluoride (10 mM NaF) prevents hemolysis by inhibition of erythrocytic glycolysis without affecting adsorption or rickettsial metabolism. Recently, rickettsial hemolysis has been shown to be associated with phospholipase A activity, which resulted in hydrolysis of fatty acids from the glycerophospholipids of the red blood cell membrane. Inhibition of either adsorption or lysis also prevented the release of free fatty acids.

Penetration by rickettsiae has many correlates with rickettsial hemolysis. Inactivation of R. tsutsugamushi by heat (56°C for 5 minutes), exposure to ultraviolet irradiation, or incubation with 0.1% formalin prevents penetration into host cells.

Penetration of L cells by R. prowazekii comprises two steps, adherence and internalization, and requires active participation by both the rickettsia and the host cell. Treatment of rickettsiae with ultraviolet irradiation, 3% formaldehyde, or 1 mM KCN inhibited adherence to and internalization into L cells. The few inactivated rickettsiae found associated with L cells were mostly adherent rather than internalized. Treatment of L cells with NaF (an inhibitor of metabolism), N-ethylmaleimide, or cytochalasin B inhibited internalization of rickettsiae. Inoculation of R. prowazekii onto L cells at large multiplicities of infection induced immediate cytotoxicity. This cytotoxic effect was associated with phospholipase A activity and hydrolysis of fatty acids from host cell phospholipids. Cytotoxicity and phospholipase activity were inhibited in a parallel manner by KCN, N-ethylmaleimide, NaF, and low temperature.

Further studies in our laboratory have extended phospholipase as a pathogenic mechanism to the plaque model, which more closely mimics actual infection, and to a member of the spotted fever group, R. rickettsii. Chemical agents which have a sound theoretical basis of inhibiting rickettsial penetration either at the step of adsorption of the rickettsia to the host cell (Amphotericin B and digitonin) or at the step of internalization associated with phospholipase A activity have been demonstrated to reduce plaque formation. Amphotericin B and digitonin have been reported to inhibit the attachment of R. prowazekii to erythrocytic cell membranes by binding to a cholesterol receptor in the membrane. Amphotericin B was introduced in concentrations of 5 and 10 µg/ml to the overlay after the establishment of infected foci on day 4 after inoculation of R. rickettsii. In order to maintain active levels of this drug which has a decay of 50% per 24 hours at 37°C, Amphotericin B was replenished in sequential overlays on days 5 and 6. On day 6 Amphotericin B caused plaque reduction of 42-45% at both concentrations. More plaques appeared on day 7 with plaque reduction of 16-23%. A similar experiment with digitonin at the same concentrations resulted in similar plaque reduction on day 6 at both concentrations (38-40%). Plaque reduction was not observed on day 7. These results demonstrate that when the levels of cholesterol receptor-binding drugs are maintained plaque reduction can be demonstrated. This suggests that inhibition of rickettsial adsorption delays the cytopathic effect of R. rickettsii in primary chick embryo cells.

Phentermine is a drug which has been shown to have phospholipase A₂ inhibitory activity. A dose response study with this drug was performed in the plaque model. Plaque reduction was demonstrated at all doses of phentermine: 69% plaque reduction at 0.5 mg/ml; 54% at 0.1 mg/ml; 25% at 0.05 mg/ml; and 32% at 0.01 mg/ml. These results demonstrate that phentermine reduces the cytopathic effect of R. rickettsii and suggest that phospholipase activity may be a pathogenic mechanism for R. rickettsii. These data extend and support the observations of Winkler that phospholipase activity is associated with hemolysis and immediate cytotoxicity of a large inoculum of R. prowazekii.

Previous reports have documented that R. conorii forms distinct plaques similar to those of R. rickettsii in the plaque model. McDade et al produced distinct plaques with R. conorii in

chick embryo cells with a first overlay of medium 199 containing 5% calf serum and 0.5% agarose and a later second overlay of medium 199, no calf serum, 0.5% agarose, and 0.01% neutral red. Wike *et al* studied the critical variables in the plaque assay system for rickettsiae and also showed that R. conorii (Malish strain) produced distinct plaques in the standard chick embryo monolayer with nutrient overlay containing agarose. Thus, the plaque model offers an opportunity to examine quantitatively and predictably the pathogenic mechanisms of R. conorii in an *in vitro* system that may be manipulated experimentally to examine hypotheses such as phospholipase-mediated injury.

Because one hypothetical explanation for the apparent rarity of severe visceral involvement in BF as compared with RMSF (encephalitis, hepatitis, pneumonitis) is lower temperature sensitivity of R. conorii, we are interested in the effects of temperature on the physiology and pathogenicity of the organism. Oaks and Osterman have investigated the effects of temperature on the optimal growth of R. conorii. This species of rickettsia has an optimal range for growth in gamma-irradiated L cells of 32-38°C with inhibited proliferation at 40°C. The low rate of proliferation at 40°C might explain the minimal visceral involvement in febrile patients whose body core temperature is about 38°C and may exceed 40°C. An unanswered question is the effect of temperature on the pathogenic mechanism of R. conorii.

Approach to the Problem

Many features of boutonneuse fever have been investigated to a far less degree than typhus fever and Rocky Mountain spotted fever. In particular, pathogenic mechanisms of R. conorii, immune mechanisms against R. conorii, and the laboratory diagnosis of boutonneuse fever have not been investigated sufficiently.

The localized lesion at the site of the tick bite, the eschar or tache noire, offers an excellent opportunity to extend our knowledge of pathogenic mechanisms, immune mechanisms, and laboratory diagnosis of BF in humans. In contrast to typhus and Rocky Mountain spotted fever in which the lesions, although numerous and widespread, are extremely focal, the tache noire is sufficiently large and contains a large contiguous network of severely injured blood vessels that will allow predictable sampling and qualitative and quantitative analysis of rickettsial infection, host cell injury, and host inflammatory and immune cellular response. Thus, although the brightfield microscopic lesions are better described in typhus fever, Rocky Mountain spotted fever and scrub typhus than in boutonneuse fever, these reports are not quantitative, often do not demonstrate rickettsiae with the efficiency and specificity of immunohistochemical techniques, and do not evaluate the ultrastructure of the human lesions. Surgically excised, well-fixed eschars should allow these studies in boutonneuse fever.

As yet no significant *in vivo* ultrastructural study of the human host-rickettsial relationship has been reported. There are two major reasons: 1) the infection in human skin is extremely focal, in the exact center of the maculopapular rash of RMSF and typhus and, thus, is difficult to find by electron microscopy; 2)

intensely infected visceral tissues from fatal cases of RMSF and typhus are not suitable for ultrastructural investigation because of postmortem autolysis that occurs prior to performance of the necropsy. Surgical biopsy of the tache noire of BF should provide well-preserved lesions containing intense R. conorii infection for ultrastructural investigation. A report of the ultrastructural aspects of an eschar in Rocky Mountain spotted fever described rickettsiae in the lesion. However, the published electron micrographs were of poor quality, and no rickettsiae were identifiable in them. Correspondence with the authors directly in an attempt to obtain copies of the original electron micrographs or the EM grids for examination personally has not been answered.

Ultrastructural studies of experimental animals in our laboratory and others have shown some of the qualitative aspects of the rickettsia-host interaction. Our ultrastructural analysis of rickettsial infections demonstrated R. rickettsii in endothelium, vascular smooth muscle, and phagocytes of infected guinea pigs in three investigations, saline-hydration prolonged survival, antilymphocyte-mediated immunosuppression, and tetracycline-treated rickettsia clearance.

A sample of the tache noire is collected by sterile skin biopsy technique under local anesthesia after obtaining the patient's informed consent. The specimen is divided into three small 1 mm³ blocks and fixed for electron microscopy by immersion in cold buffered glutaraldehyde-formaldehyde solution. The fixed specimen may be held in this solution for the period of shipping from Italy to our laboratory. On arrival at the Infectious Pathogenesis Laboratory in the Department of Pathology of the University of North Carolina, the specimen will be postfixed in 1% osmium tetroxide, dehydrated in graded alcohol concentrations, embedded in a mixture of Epon and Araldite, ultrathin sectioned on an ultramicrotome, and stained with uranyl acetate and lead citrate. Sections will be examined on a high resolution Zeiss 10 A electron microscope. Other electron microscopes including a JEM 100B and a high resolution scanning electron microscope are also available within the departmental facilities should the need arise.

The remainder of the specimen is fixed in neutral buffered-4% formaldehyde for routine histology, histochemistry, and immunohistochemistry. Fixed tissue will be embedded in paraffin and a ribbon of serial sections will be cut at 4 μ m thickness. Adjacent sections will be mounted for staining by hematoxylin-eosin (H & E) for routine evaluation of pathologic lesions, by phosphotungstic acid-hematoxylin (PTAH) for fibrin thrombi, by Voerhoff-van Gieson technique (VV) for evaluation of integrity of vascular elastic tissue, by modified Brown-Hopps (BH) technique for histochemical demonstration of rickettsiae, and by Giemsa technique and methyl green pyronin (MGP) for identification of host immune and inflammatory cells. Among these stains, PTAH and VV yield highly sensitive results, BH demonstrates intracellular rickettsiae but with less sensitivity, consistency, and specificity than immunofluorescence, and Giemsa and MGP assist in identification of eosinophils, basophils, neutrophils, activated lymphocytes, and plasma cells but leave a large portion of unidentified mononuclear cells which include macrophages and some populations of lymphocytes.

Adjacent sections from the ribbon are processed for

immunofluorescent demonstration of R. conorii. Sections are affixed onto clean glass slides with nonautofluorescent LePage Bond Fast Resin Glue to prevent them from being washed off the slide after digestion with trypsin. Sections affixed to slides with glue are heated in an oven at 60°C for 1 hour, deparaffinized in three changes of xylene for 10 minutes each, and rehydrated through serial changes of ethanol in concentrations of 100%, 95%, 70%, 50%, and 35% and finally in distilled water. Sections are then incubated in 0.1% trypsin with 0.1% CaCl₂, pH 7.8, at 37°C for 4 hours. The slides are washed thoroughly in distilled water, washed for 30 minutes in phosphate-buffered saline, and reacted with the specific immunofluorescent system for R. conorii. We have used anti-SFG rickettsial conjugate in the direct immunofluorescence system and indirect immunofluorescence with guinea pig immune anti-R. conorii serum followed by anti-guinea pig immunoglobulin conjugate to demonstrate structures which have the expected vascular location and coccobacillary morphology of rickettsiae.

Studies of pathogenic mechanisms of R. conorii are performed in the plaque model which we have exploited in investigations of pathogenic mechanisms of R. rickettsii. Aliquots of R. conorii stock are thawed and diluted in sucrose phosphate buffer to contain 500 pfu per ml. Confluent monolayers of Vero cells are inoculated with either 0.1 ml of diluted rickettsial stock containing 50 pfu of R. conorii or uninfected diluent. Rickettsial plaque technique is performed according to the method of Wike and Burgdorfer and Wike et al. After 30 minutes for absorption to occur and penetration to begin, monolayers will be overlaid with 4 ml of 0.5% agarose in minimum essential medium with 5% fetal bovine serum and incubated at 35°C. On day 4 after inoculation, 4 ml of second overlay with 1% neutral red is added, and the flasks are allowed to incubate in the dark at 35°C. Flasks are examined daily for plaques afterwards with observations of monolayers by inverted microscope and with collection of specimens for examination by immunofluorescent and transmission electron microscopy.

The sides of the 25-sq cm Falcon flasks opposite the monolayers are removed by cutting the plastic. Agarose gel overlays are gently removed by separating the overlay from the sides of the flask with a sharp spatula edge and allowing the gel to detach under the force of gravity. Exposed monolayers are fixed in 70% ethanol for 20 minutes prior to direct immunofluorescent staining for R. conorii with a specific anti-R. conorii conjugate. Following incubation of monolayers with conjugate for 30 minutes, they are washed in phosphate-buffered saline for 30 minutes, washed in distilled water, and mounted with 90% glycerol in phosphate-buffered saline (pH 9) and cover glass. Monolayers are examined on a Leitz Ortholux ultraviolet microscope equipped with incident beam illumination and barrier and exciter filters for fluorescein isothiocyanate fluorescence microscopy.

Monolayers with overlays removed as described for immunofluorescence are fixed by covering the cells with a solution of buffered 2.5% glutaraldehyde for 1 hour. Cells are maintained on the plastic surface throughout postfixation in osmium tetroxide, dehydrated through a graded series of ethanol and hydroxypropyl methacrylate solutions, and embedded in Mollenhauer's Epon-araldite No. 2. following polymerization in an oven at 37°-45°C for 24 hours and then 60°C overnight. Embedded monolayers are separated

from the plastic flasks. At this point, rickettsial plaques may be observed with the unaided eye as distinct clear zones surrounded by a grey-black carpet of cells. Plaques and adjacent cells are cut out and reembedded in flat molds with the monolayer perpendicular to the plane of sectioning. Ultrathin sections are cut on an LKB ultramicrotome using a diamond knife. Observation of the block during sectioning reveals the exact relationship of the section to the plaque. Sections are stained with lead citrate and uranyl acetate and examined on a Zeiss 10A transmission electron microscope.

Plaque size, time of appearance and phase contrast morphology are observed. The relationship of R. conorii to plaques is observed by immunofluorescence microscopy. The cytopathology of injured cells is described including state of rough endoplasmic reticulum, mitochondria, plasma membrane, and nucleus. For each plaque zone (center, margin, and periphery), the quantity of R. conorii in cytopathologic and cytologically normal cells will be counted and subjected to statistical analysis for association or non-association of cytopathology with intensity of intracellular infection. The cytology of uninfected cells adjacent to the plaque will be examined.

Plaque model studies of penetration-associated pathogenic mechanism employ the plaque model, Amphotericin B (5 and 10 μ g/ml), and digitonin (5 and 10 μ g/ml), compounds which have been shown to bind to cholesterol-containing membrane receptors, to block attachment of R. prowazekii to erythrocyte plasma membrane and to reduce plaque formation by R. rickettsii which are incorporated into the agarose-nutrient medium overlay. The second overlay contains the same concentration of the cholesterol-receptor binding drug. Plaques are enumerated at the time of appearance of distinct plaques in untreated plaque assays of the same inoculum for statistical analysis. In a second set of similar experiments Amphotericin B and digitonin are introduced only in the second overlay on day 4 after inoculation, at which time infected foci will be well established. Significant plaque reduction in this experiment indicates blocking of a pathogenic mechanism, not just abortion of initial infection.

Another experiment is designed to inhibit the penetration-associated mechanism by inhibition of phospholipase activity. The approach is to utilize the phospholipase inhibitor, phentertine, in doses which we have shown not to adversely affect the monolayer yet to cause reduction in R. rickettsii plaques. Concentrations of phentertine of 0.5, 0.1, 0.05, and 0.01 mg/ml are incorporated in the agarose-nutrient medium overlay. Comparison by statistical analysis of plaque counts from each dose and untreated monolayers with the same inoculum of R. conorii yield a dose-response curve that indicates whether phospholipase activity is necessary for the expression of cell injury by R. conorii.

One hypothesis which can be tested in the plaque model is that the paucity of signs and symptoms pointing to visceral involvement is due to a lower threshold of temperature sensitivity of R. conorii. The inability to produce pathogenic effects at temperatures greater than 38°C could explain the relative lack of severity of BF when compared with RMSF despite the 91-94% relatedness of the etiologic agents. The plaquing efficiency of various strains of R. conorii and R. rickettsii are compared at

32°C, 34°C, 36°C, 38°C, and 40°C. Variation in number of plaques formed, plaque size (area measured morphometrically by computer assisted image analysis), and time of onset are examined. These results reflect the effect of temperature on pathogenic effects.

The hypothesis of secretion of a potent extracellular toxin by R. conorii can be examined in an experiment utilizing parabiologic tissue culture chambers. Parabiologic chambers containing cell monolayers are separated by a filter with 0.22 μ pore size. This filter prevents the passage of rickettsiae from one chamber to the adjacent chamber, but allows free passage of macromolecules such as metabolites, putative toxic products, or enzymes. In some pairs of chambers, one chamber is inoculated with 10 plaque-forming units of R. conorii. Other pairs of chambers are maintained with both chambers uninoculated as controls. On days 3, 5, and 7 postinoculation, trypan blue is added to selected pairs of chambers, and selected chambers are examined by immunofluorescence for R. conorii. The degree of cell injury in infected chambers, uninfected-rickettsial products exposed chambers, and control chambers is evaluated blindly by estimation of percentage of cells failing to exclude trypan blue. Immunofluorescence for R. conorii confirms the limitation of infection to inoculated chambers and allows estimation of the percentage of the monolayer that is infected.

Results

The study of taches noires from patients with boutonneuse fever has been performed in collaboration with physicians at the University of Palermo. This collaboration has proven successful with opening of several avenues for the continued investigation of the pathology, pathophysiology, and clinical aspects of R. conorii infection in humans. During the period June-August 1983, I spent six weeks in Sicily as NATO Visiting Professor of Tropical and Subtropical Diseases. My major collaborators were Professor Mansueto for clinical studies of boutonneuse fever and Professor Tringali for rickettsial studies. Twenty patients with clinical boutonneuse fever under the care of Dr. Barba at the Guadagna Infectious Disease Hospital were investigated. During the visiting professorship, seven biopsies of taches noires were collected. With four additional tache noire biopsies collected prior to my arrival and after my departure, 11 patients' taches noires were biopsied in all during 1983. Some patients with taches noires were not biopsied principally for reasons of location related to cosmetic concern or proximity to vital structures, e.g., carotid artery. Other patients had boutonneuse fever without a tache noire. Numerous personal contacts were developed which should provide the basis for future investigation of the pathology of the tick bites (Professor Tosti, a dermatologist-dermatopathologist) and the role of the tick bite in the pathogenesis of the tache noire. Several clinical studies that are not a part of this contract should prove interesting for correlation with the tache noire investigation. These include evaluation of specific serum proteins and polypeptides that are related to the inflammatory process (some as acute phase reactants) and hemolysis: alpha-1-antitrypsin, C-reactive protein, C3, C4,

haptoglobin, and prekallikrein and evaluation of hepatic injury. Rickettsia conorii was isolated from patients by inoculation of guinea pigs with acutely collected blood or plasma samples. Intradermal inoculation resulted in formation of an eschar at the site of inoculation in many animals. Guinea pig eschars were demonstrated to contain R. conorii by direct immunofluorescence with the anti-spotted fever group rickettsia conjugate obtained from the Centers for Disease Control. The collaborative relationships with Drs. Mansueto, Tringali, and others is a valuable and productive resource for the study of R. conorii and boutonneuse fever. They are very interested in the scientific questions related to the tache noire, boutonneuse fever, and R. conorii. They have made great efforts to obtain clinical material, conduct complete patient laboratory diagnosis confirmation and clinical followup, and establish a rickettsiology laboratory. Ongoing and future investigation of pathogenetic mechanisms, diagnostic methods and possibly preventive measures are mutually achievable goals. The clinical and professional resources available in Sicily are valuable; they comprise interdependent personal and scientific relationships in a setting which offers excellent opportunities for completion of designed investigations. Dr. Mansueto and Dr. Tringali have a strong commitment to these studies, and they command an impressive ability to direct their staffs and follow their patients in the classic European style that brings about a thorough completed study.

Four studies of patients with boutonneuse fever in Sicily were initiated prior to the beginning of this research contract. They were completed and prepared for publication during the first year of this project. In the first study, the method for demonstration of R. conorii in infected tissues fixed in formalin and embedded in paraffin utilizing the deparaffinization, trypsin digestion method and immunofluorescence was described. T-lymphocyte deficient, nude mice were inoculated cutaneously with 4.8×10^4 plaque forming units of R. conorii (strain 7) and were sacrificed on days 3, 7, and 15 after inoculation. An adult guinea pig was inoculated intradermally with the same inoculum and sacrificed on day 11. Skin from the inoculation site, spleen, and liver of the mice and skin from the inoculation site eschar of the guinea pig were collected and fixed in 4% neutral buffered formaldehyde. A biopsy of the rash from a Sicilian patient with boutonneuse fever was obtained on day 6 after onset of fever and was similarly processed. Sections at 4 μ m thickness were affixed to slides with LePage Bond Fast Resin glue (LePage, Ltd., Montreal, Canada) and were incubated in an oven at 60°C for 1 hr., deparaffinized in three changes of xylene for 10 min each, rehydrated through serial changes of ethanol in concentrations of 100%, 95%, 70%, 50%, 35%, and finally in distilled water. The slides were then incubated for 4 hrs in a 0.1% trypsin (Grand Island Biological Co., Grand Island, NY) with 0.1% CaCl_2 , pH 7.8 at 37°C. After incubation slides from the patient and from mice were washed thoroughly in distilled water, washed for 30 min in PBS, and allowed to react for 30 min with anti-R. conorii guinea pig serum prepared in our laboratory, washed for 30 min in PBS, and incubated with specific anti-guinea pig immunoglobulin FITC conjugate (DAKO) for 30 min, washed in PBS for 30 min, rinsed in distilled water, mounted in a solution of glycerol in PBS, and examined by ultraviolet microscopy with use of

FITC barrier and exciter filters. For guinea pig tissues we used anti- R. conorii human serum and an anti-human conjugate from DAKO.

Furthermore, we submitted all the slides to similar procedures but using direct immunofluorescence with a rabbit immunoglobulin FITC conjugate prepared against the antigens of R. rickettsii at the Centers for Disease Control, Atlanta, Georgia, as described previously by Walker and Cain.

Organisms with morphology of rickettsiae were observed predominantly in the vascular walls in the endothelial location in clusters or arranged end-to-end. Only smaller quantities were observed away from the vessel walls, apparently associated with the predominantly macrophagic perivascular cellular reaction. These organisms were demonstrated by both indirect immunofluorescence and direct immunofluorescence. An observation deserving comment is the better results obtained with the direct reaction using rabbit anti- R. rickettsii conjugate. Hebert et al have shown that this serum is specific for the spotted fever group of rickettsiae. It is a well known, high-titered, well standardized reagent that gives consistently excellent results. It reacts with R. conorii and the titer with this agent is only one dilution below the titer with R. rickettsii. In our hands it gives good, clean preparation. The indirect test was performed with guinea pig or human anti- R. conorii whole serum, both with relatively low titers. It is known that indirect immunofluorescence gives a certain degree of nonspecific fluorescence. Indeed, in our preparations there was some of this nonspecific fluorescence, and sometimes we had to rely on the location, size, and morphology of the fluorescent structures to consider them to be rickettsiae. In a second publication, the histologic lesions of the tache noire were described and organisms of R. conorii were demonstrated in 5 of the 6 biopsies using the deparaffinization-trypsinization immunofluorescent technique.

One of these patients is described in detail in a case report in the American Journal of Tropical Medicine and Hygiene because of the documentation of the mild end of the clinical spectrum of human R. conorii infection that he represents. A sixty year old agricultural laborer from Sciacca, Italy noted the presence of a tick on the lateral aspect of the right lower leg and removed it on March 10, 1982. Twenty days later he sought medical attention because of the development of a skin lesion at the site of tick bite. The cutaneous lesion consisted of a central, black ulcer 2 cm in diameter surrounded by vesicles and a peripheral erythematous zone. Indirect immunofluorescent antibody assay confirmed the diagnosis of BF with anti- R. conorii IgG titer of 1:320 and IgM titer of 1:80.

On April 3, 1982 a biopsy of the tache noire was performed. Brightfield microscopy revealed foci of pseudoepitheliomatous hyperplasia in the epidermis surrounding the necrotic mass of karyorrhectic debris, fibrin and keratin, corresponding to the eschar. In the surrounding dermis and underlying subcutaneous tissue, there were perivascular accumulations of macrophages, lymphocytes, and numerous eosinophils. The vascular endothelial cells were swollen. Examination of sections by the method of deparaffinization and trypsin digestion followed by direct immunofluorescence with a conjugate reactive with spotted fever group rickettsiae showed focal clusters of coccobacillary organisms in the lining of the vessel walls in the reticular dermis. The

immunofluorescent conjugate was prepared at the Centers for Disease Control using killed R. rickettsii as antigen for immunization of rabbits. The conjugate of the globulin fraction of rabbit antiserum has also been demonstrated to react with R. conorii at a titer of 1:512.

Reaction of sections of the eschar with guinea pig pre-serum by indirect immunofluorescence using a 1:20 dilution of serum and 1:40 dilution of rabbit anti-guinea pig IgG conjugate (DAKO, Accurate Chemical and Scientific Corporation, Westbury, N. Y.) revealed no organisms whereas the same indirect immunofluorescent system using convalescent serum collected from the same animal one month after inoculation of R. conorii revealed foci of thin bacilli compatible with rickettsiae.

On April 6 the anti- R. conorii titers remained at the same level while both the third and fourth components of complement were slightly elevated. At no time during his course did the patient report a fever. He was afebrile and did not have a rash during this evaluation of the eschar or during the following eight months.

The observation of spotted fever group rickettsiae at the site of tick bite is strong evidence for inoculation of rickettsiae into the skin by tick bite, colonization of vascular endothelium by the rickettsiae, and stimulation of host defenses without any systemic signs or symptoms of disease.

The two principal hypotheses that may explain the occurrence of R. conorii infection manifested only by an eschar are either that the strain of R. conorii-like rickettsia was of relatively low virulence or that previous spotted fever group rickettsial infection provided partial immune protection. Investigation of spotted fever group rickettsiae in North America has revealed a great diversity of rickettsial species with a spectrum of virulence as judged by response of guinea pigs to inoculation. At the present time the range of virulence of R. conorii and possibly other spotted fever group rickettsiae in the Mediterranean basin is not known. The serologic documentation of infection with R. conorii among as many as 20% of persons in western Sicily who are engaged in agricultural activities and give no history of BF suggests that there are nonpathogenic strains of R. conorii in Sicily. The possibility of a previous infection with R. conorii cannot be excluded. The serology, in fact, demonstrated a higher level of antibody to R. conorii in the IgG class than the IgM class, as would be expected in an anamnestic immune response. Bourgeois *et al* have shown that in primary infection with R. tsutsugamushi the antibody response is mainly of the IgM class. In contrast, reinfection scrub typhus stimulates an antibody response mainly of the IgG class. We have also observed these two types of antibody response to R. conorii in BF. It may be hypothesized that our patient, an agricultural laborer who is at high risk for tick bite and/or R. conorii infection, had a prior infection with residual immunity sufficient to contain the subsequently inoculated organisms at the portal of entry. Elucidation of the host-rickettsia relationship between humans and R. conorii will require extensive investigation of strains of R. conorii isolates in Sicily and of the immunology of human host defenses against rickettsiae. BF offers an excellent opportunity for the advancement of knowledge concerning rickettsial pathogenesis and immunity.

The fourth manuscript on R. conorii infection in vivo prepared using data collected prior to the start of this contract presents a study of infection of genetically immunodeficient mice with R. conorii. In order to determine the definitive importance of T- and B-lymphocytes in immunity to Rickettsia conorii, mice genetically deficient in T-cells, B-cells, or both T- and B-cells were infected experimentally. T-lymphocytes rather than humoral antibodies were crucial to rickettsial clearance and a reduced mortality rate. Mice incapable of an antibody response to polysaccharide capsular antigens effectively controlled rickettsial infection with no mortality. In contrast, nude mice produced antibody to thymus-independent antigens early in the course of infection, yet experienced severe rickettsial infection with deaths occurring.

These experiments in genetically immunodeficient mice document the importance of T-lymphocytes in the clearance of R. conorii from the tissues of infected mice. On day 7 the spleens and livers of T-cell deficient and T- and B-cell deficient mice contained numerous rickettsiae, whereas animals with an intact T-lymphocyte immune system contained very few rickettsiae in their hepatic and splenic tissues. The only animals that died as a result of R. conorii infection in these studies were those deficient in T-lymphocytes whether or not they had B-lymphocytes. These results support the conclusion of Kokorin et al that T-lymphocytes confer protection against R. conorii infection in mice. Moreover, the generation of a humoral immune response in our experiments did not correlate with clearance of rickettsiae and protection from death. T-lymphocyte deficient nude mice synthesized antibodies to R. conorii detectible on day 7 at which time the visceral organs contained many rickettsiae. Despite the presence of antibodies in these animals, mortality was observed. Nevertheless, B-lymphocyte deficient mice had no antibodies and yet had effectively restricted rickettsial proliferation in the liver and spleen on day 7; moreover, none of these mice died. These observations conform to the conclusion of previous studies on immunity to members of the genus Rickettsia, namely that T-lymphocytes are crucial for immunity.

The immune response has not been shown in this or previous studies to be a pathogenic mechanism of tissue injury in infections by members of the genus Rickettsia. The cytolytic effect of lymphokines on rickettsia-infected cells in vitro again raises the question of an immunopathologic mechanism of tissue and cellular injury in rickettsial diseases. Although this investigation does not exclude a contribution to cell injury by lymphokine-mediated cytolysis, it does document that in the overall balance in vivo the T-lymphocyte affords protection against R. conorii. Other studies including the in vitro plaque model have demonstrated that rickettsiae possess direct cytopathic activity that appears to be mediated at least in part by the phospholipase- associated penetration mechanism and is active in the absence of the immune system.

The similarity of the hepatic lesions of these animals to hepatic lesions in boutonneuse fever contributes to understanding of the pathogenesis of the human lesions of multifocal hepatocellular necrosis and associated focal hepatic inflammatory response. The observation of R. conorii in these lesions in mice

suggests that the rickettsiae play an important role in their pathogenesis. The immunodeficient mouse model of R. conorii infection should be useful in further dissection of pathogenic mechanisms of hepatic injury by R. conorii and in evaluation of the importance of stimulation of each component of the immune system by specific purified rickettsial antigens in protective immunity and vaccine design.

The study of Sicilian taches noires has progressed substantially (Table 1). Eleven taches noires were examined this past year under the first year of this research contract for a total of 17 since the beginning of the collaborative study with Dr. Mansueto. The principal features of these biopsies are severe vasculitis with a predominance of small lymphocytes and large mononuclear cells (compatible with stimulated lymphocytes and macrophages) and ischemic cutaneous necrosis. Thrombosis is a minor feature, less impressive than perivascular edema. R. conorii has been identified in 11 of 17 cases, and immunofluorescence of the tache noire appears to be an effective diagnostic test prior to or during the first 1-2 days of antirickettsial drug treatment. Tissues from seven biopsies have been embedded for electron microscopy and will be sectioned and examined soon. Most of these patients' diagnoses have been confirmed by indirect immunofluorescent antibody test or isolation of rickettsiae although serologic and rickettsial isolation data have not yet been received from Palermo on a few patients. The prospects of this work providing answers to questions regarding pathogenesis and immunity to R. conorii appear quite good.

In general visceral involvement is considered to be uncommon in boutonneuse fever. Although moderate elevation in serum lactate dehydrogenase is frequently observed, physical clinical evidence for important visceral pathology is unusual. Several studies of selected patients with boutonneuse fever have documented elevated hepatic enzymes and focal hepatic microscopic lesions. A series of eight patients from Sicily with boutonneuse fever was examined with serologic documentation of the diagnosis by indirect immunofluorescent antibody in seven patients, hepatomegaly in five patients, and peak SGOT of 50-70 and peak SGPT of 34-123 in five patients. The biopsies, which were obtained late in the course of illness, (day of fever ranged from 7- 23, average 12.8), showed no lesions. Considering the mild involvement, focal nature of the lesions, small sample obtained by needle biopsy, and opportunity for repair prior to biopsy late in the course, we were not surprised with the results. In a subsequent study of needle biopsies of four patients observed last summer, lesions were identified in all cases. These patients were biopsied during the acute phase of boutonneuse fever (Table 2). The microscopic lesions are similar:

Patient 1. There was only a small fragment of liver on the slide. It contained fatty change, focal hepatocellular necrosis (pyknotic cells), focal inflammation, ballooning swelling of hepatocytes, and a mitotic figure. The presence of all three stages: necrosis, inflammatory cell response, and mitotic regeneration suggests an ongoing injury of long enough duration for a host response and host regenerative activity.

Patient 2. There was focal hepatocellular necrosis and focal inflammatory cellular accumulation. Samples of the biopsies from

Table 1
Evaluation of Iaches Noires from Sicilian Patients

Patient	Cutaneous Necrosis	Vasculitis	Thrombosis	Dermal Edema	Predominant ^a WBC	IF ^b R. conorii	EM ^c	Lab Confirm Diagnosis
1	+++ ^d	+++	+	++	L,M	+		+
2	0	+++	0	++	L,E	+		+
3	+	+++	0	++	L,M	+		+
4	++	+++	++	++	L,M	+		+
5	0	+++	+	++	L,M	+		+
6	0	++	0	++	L	0		+
7	0	++	+	+	L	+		+
8	+++	++	+	++	L	+	+	+
9	0	++	0	++	L	0	+	+
10	+++	++	0	++	L	0	+	+
11	+++	+++	0	++	M	0	+	+
12	+++	+++	+	+++	L,M	?	+	+
13	+++	+++	+++	++	L,P	+	+	+
14	+++	+++	0	+	L	+	+	+
15	+++	+++	NA ^e	NA	NA	0		
16	+++	+++	0	+	L	+		+
17	NA	++	0	++	L	+		+

a - Predominant perivascular leukocytes: L, small lymphocyte; M, large mononuclear cell; E, eosinophil; P, PMN
b - Presence (+) or absence (-) of immunofluorescent Rickettsia conorii
c - Tissue embedded for electron microscopy (+)
d - Absent (0); +, mild; ++, moderate; +++, severe
e - Feature not available for evaluation

Table 2

Patients with Boutonneuse Fever Examined by Hepatic Biopsy in the Acute Phase

Patient	Day of Bx ^a	Day of Tick Bite ^b	<u>Tache noire</u> ^c	IF ^d	Lesions ^e
1	3	-2	+	-	+
2	1	0	-	-	+
3	5	unk ^f	-	-	+
4	7	unk	+	-	+

a - Number of days of fever prior to biopsy

b - Number of days between observation of attached tick and onset of fever

c - Presence (+) or absence (-) of tache noire

d - Immunofluorescent demonstration of Rickettsia conorii in liver, (-) negative

e - Presence of microscopic lesions

f - Unknown

patients 2, 3, and 4 have been embedded for electron microscopy.

Patient 3. There was focal necrosis and inflammatory cellular response.

Patient 4. Multifocal necrosis and inflammatory cellular response resemble the lesions described by Guardia *et al* (The liver in boutonuse fever. *Gut* 15: 549-551, 1974). These lesions were not true granulomas. The necrosis apparently preceded the resulting focal inflammatory reaction.

Samples of the biopsies from patients 2, 3, and 4 have been embedded for electron microscopy. Examination of needle biopsies from four patients sent by Dr. Faure in Grenoble, France, who has reported the hepatic histopathology of boutonuse fever, also revealed no immunofluorescent *R. conorii*.

Severe human *R. conorii* infections have been reported recently in South Africa, France, and Israel. Correspondence with two of the authors of these reports has resulted in the opportunity to evaluate the pathology of severe and fatal *R. conorii* infection including two necropsies. The pathology and distribution of *R. conorii* in fatal cases has not been reported previously. Dr. Gear, an eminent senior rickettsiologist from South Africa, reported two fatal cases and a severe case with digital gangrene. He has shipped to our laboratory material from the gangrenous digits, from the necropsy of one of the fatal cases, and from the necropsy of a subsequent fatal case, all documented as *R. conorii* infections. Dr. Raoult from France did not have necropsies on his cases but agreed to send a liver biopsy from a patient with boutonuse fever.

Paraffin sections of tissues from the three cases of South African tick bite fever were stained by routine hematoxylin-eosin method, phosphotungstic acid-hematoxylin technique, and deparaffinization-trypsin digestion-immunofluorescence method with a conjugate reactive with *R. conorii* as reported in detail previously. Tissues examined were cerebrum, cerebellum, kidney, liver, spleen, heart, lung, lymph node, and adrenal gland (case 1); cerebrum, kidney, liver, spleen, heart, lung, and pancreas (case 2); and gangrenous fingers (case 3). Positive control tissues for *R. conorii* immunofluorescence included eschar from guinea pig inoculated intradermally with *R. conorii* (strain 7), eschars from patients with serologically documented boutonuse fever, and liver tissue from nude mouse inoculated with *R. conorii* (strain 7).

Thin bright green immunofluorescent bacilli were observed only in the location of vascular endothelium and of macrophages in hepatic and splenic sinusoids and lymph node sinuses. Nonspecific staining of host tissues was not seen.

In case 1 the brain contained numerous rickettsiae in cerebral and cerebellar blood vessels and foci of rickettsiae in the subarachnoid location of the leptomeninges. Rickettsiae were observed in glomerular arterioles, capillary tufts, intertubular blood vessels, and arterial endothelium. In the liver rickettsiae were identified only in a few sinusoidal lining cells; no rickettsiae were seen in hepatocytes. Splenic rickettsiae appeared to be in macrophages and arteriolar endothelium. Few foci of *R. conorii* were observed in capillaries between myocardial cells, pulmonary alveolar septa, and macrophages within marginal and draining sinuses of a lymph node. No rickettsiae were demonstrated in adrenal gland.

In case 2, numerous rickettsiae infected cerebral blood

vessels, glomerular arterioles and capillary tufts, renal arterial endothelium, and intertubular blood vessels near the corticomedullary junction. Few hepatic rickettsiae were observed in scattered sinusoidal lining cells. In spleen, rickettsiae were identified in arteriolar endothelium and macrophages in small quantities and in one medium sized artery in a large amount. In the heart, a few foci of rickettsiae were present in arterial endothelium and capillaries between myocardial fibers. Very few rickettsiae infected pulmonary alveolar capillaries. Small foci of rickettsiae were seen in capillaries and septal blood vessels of the pancreas and blood vessels in the dermis of skin.

In case 3, several foci containing numerous *R. conorii* were identified in the injured blood vessels at the margin between viable and necrotic tissue. Rickettsiae were not observed in the mummified necrotic zone or in the zone of healthy tissue.

In case 1 the cerebrum and cerebellum contained numerous foci of perivascular mononuclear cells, some of which infiltrated the adjacent neuropil. There was widespread enlargement of endothelial cells, and a diffuse mild increase in microglial cells infiltrating neuropil. The subarachnoid space had a mild infiltration of mononuclear cells and a focal small hemorrhage with erythrophagocytosis by macrophages. The kidney was severely autolytic, but multiple foci of mononuclear leukocytic vasculitis could be identified in the outer part of the medulla near the corticomedullary junction. Hepatic lesions included multifocal, randomly distributed coagulative necrosis of solitary hepatocytes, few polymorphonuclear leukocytes and moderate quantities of small lymphocytes in portal triads, mild steatosis, moderate congestion, and mild sinusoidal leukocytosis. There were no granulomas, portal vasculitis, or leukocytic accumulation around necrotic hepatocytes. Matching of serial sections by brightfield microscopy and immunofluorescence demonstrated necrotic hepatocytes adjacent to infected sinusoidal lining cells and *R. conorii* within splenic arterioles that contained thrombi and karyorrhectic debris. The red pulp was congested, and no germinal centers were observed. The myocardium contained a few foci of interstitial leukocytes, predominantly mononuclear cells. Lung lesions included protein-rich pulmonary edema, congestion, focal nonocclusive thrombosis, focal acute pneumonia with alveolar polymorphonuclear leukocytic exudate, and foci of fibrosis containing carbon pigment and birefringent silica crystals. Lymph node also contained anthrosilicotic fibrosis. There were foci of adrenocortical necrosis with surrounding leukocytic response, and two periadrenal arteries had foci of acute vascular wall necrosis.

In case 2, many foci of vasculitis in the brain consisted of mononuclear leukocytes infiltrating the blood vessel wall and surrounding neuropil. There was also a mild mononuclear leukocytic leptomeningitis. Lesions in the severely injured kidney included karyorrhexis, thrombosis, and leukocytic infiltration of glomerular arterioles, karyorrhexis in glomerular capillary tufts, cortical vasculitis with perivascular mononuclear leukocytes, plasma cells, and focal hemorrhage, multifocal cortical interstitial edema, and multifocal severe vasculitis with petechiae at the corticomedullary junction and in the outer part of the medulla. The liver contained multifocal necrosis of solitary hepatocytes, intracanalicular cholestasis, mild steatosis, hepatocellular giant cell

transformation, and mitosis. No granulomas, portal vasculitis, or leukocytic response to necrotic hepatocytes were observed. The spleen had a capsular hemorrhage and polymorphonuclear leukocytes and plasma cells in the red pulp. The lungs were congested with intraalveolar amorphous eosinophilic material compatible with pulmonary edema, scattered intraalveolar erythrocytes, and deposits of carbon pigment. The pancreas was autolyzed, but contained a focus of identifiable perivascular mononuclear cell infiltrate. The skin showed multifocal dermal and subcutaneous vasculitis with perivascular edema and leukocytes including polymorphonuclear leukocytes. Two dermal blood vessels contained thrombi that did not occlude the lumina.

In vitro experiments on the pathogenic mechanism or mechanisms of R. conorii have employed the parabiotic chamber model and the plaque model. Parabiotic chambers were employed to determine whether any soluble rickettsial product would injure uninfected cells sharing the same culture medium with cells infected and killed by R. conorii.

The hypothesis that cell and tissue injury are mediated by a rickettsial toxin has been suggested although an exotoxin has never been demonstrated and rickettsial lipopolysaccharides do not have potent toxic activity. Much of the confusion concerning rickettsial pathogenesis is the result of the name given to the phenomenon of the lethal effect of large doses of viable rickettsiae when inoculated intravenously into mice. Traditionally, this rickettsial laboratory assay has been termed the "mouse toxin phenomenon" although it cannot be produced by rickettsiae that are metabolically inactive or dead, and this toxicity has never been produced by a purified component of rickettsiae.

Pairs of sterile parabiotic chambers (Bellco Glass, Vineland, NJ) were separated by 25 mm diameter cellulose triacetate membrane filters (Gelman Sciences, Ann Arbor, MI) with 0.2 μ m pore size sealed between the chambers with silicone stopcock grease. Coverslips measuring 10.5 x 35 mm were placed in each chamber and were seeded with 5 x 10⁵ VERO cells (CDC Tissue Culture Unit, Atlanta, GA). After incubation at 37°C in minimum essential medium with 5% heat-inactivated fetal calf serum and 10% tryptose phosphate broth for 24-48 hours, monolayers were confluent. The medium was removed, and 36-360 plaque forming units of R. conorii (strain 7) were inoculated into one chamber of parabiotic chambers. After 30-45 minutes for adsorption of inoculum, 10 ml of the same medium was added. Control pairs of chambers were not inoculated with rickettsiae. Coverslips from adjoining inoculated and uninoculated chambers were examined for evidence of cell death as determined by trypan blue staining and for presence and distribution of R. conorii by direct immunofluorescence. Uninoculated pairs of chambers were examined as controls on day 7 after inoculation. For a positive toxin control, one chamber of each of two pairs was inoculated with a fresh clinical isolate of Pseudomonas aeruginosa with examination of chambers on day 3 and on day 5 by trypan blue staining. The coverslips were examined on days 5 and 6 by phase contrast microscopy after trypan blue staining and then after acetone fixation by direct immunofluorescence for

rickettsiae.

The monolayers infected with R. conorii showed severe cytopathic effect but with less necrosis than R. rickettsii-infected monolayers although nearly all of the cells were infected. In contrast, the adjacent uninoculated monolayers appeared without cytopathic effect. Validation of the parabiologic chamber toxin model was provided by demonstration of progressive destruction of the monolayers in the chamber infected with P. aeruginosa and in the uninfected chamber when examined on days 3 and 5.

The plaque model has been useful in the investigation of the role of the phospholipase-associated rickettsial entry mechanism in cell injury by R. conorii. We have used VERO cell monolayers and R. conorii (strain 7). Two different drugs that have been reported to possess phospholipase A₂ inhibitory capability, phenthermine and indomethacin, were incorporated into the overlay media. Phenthermine was added to both the first and second overlay media in the following final concentrations: 0.1 mg/ml, 0.2 mg/ml, 0.4 mg/ml, 0.8 mg/ml, and 1.6 mg/ml. Plaques were counted on day 6 after inoculation. The results are shown in Table 3. Indomethacin was incorporated in both overlay media in final concentrations of $10^{-3}M$, $10^{-4}M$, $10^{-5}M$, and $10^{-6}M$. Plaques were counted on days 6 and 7. The results are presented in Table 4. In an attempt to determine whether the effect of one of these drugs with phospholipase A₂ inhibitory activity, phenthermine, acts on a rickettsial activity or on a host cell activity, the compound was incubated with either R. conorii or VERO cell monolayers prior to inoculation. The rickettsiae were then diluted 10^{-2} such that the final concentration of phenthermine at the time of inoculation was negligible. The pretreated monolayer was washed with 3 ml of sterile phosphate buffered saline prior to inoculation to remove unbound phenthermine. Plaques were counted on day 7 after inoculation of R. conorii. The results are shown in Tables 5 and 6. Uninfected monolayers showed no toxicity of phenthermine pretreatment over the range of 0.1 - 4.0 mg/ml concentration. There was partial toxicity at 10 mg/ml and complete destruction of the monolayer at 20 mg/ml and above.

Because recent studies in our laboratory have shown that synthetic diamidine type protease inhibitors have the ability to block plaque formation by R. rickettsii, we tested the effect of the most effective compound on the R. conorii plaque model. Bis(5-amidino-2-benzimidazolyl)methane, (BABIM), was first incorporated into both first and second overlay media at concentrations of $10^{-4}M$, $10^{-5}M$, and $10^{-6}M$. Plaques were counted on day 6 after inoculation. The results demonstrated that this inhibitor of trypsin-like proteases blocked plaque formation at $10^{-4}M$ and $10^{-5}M$ concentrations (Table 7).

R. conorii plaques have been fixed for electron microscopy, embedded in Epon-Araldite, removed from the flask while the topography of the plaque was maintained, and reembedded in Epon-Araldite for sectioning perpendicular to the plane of the monolayer. Ultrathin sectioning, staining, and ultrastructural analysis of the host-rickettsia relationship in R. conorii plaques should be achieved during the second year of the contract. Additional experiments which have been begun, but have not yielded significant results, include the effect of hemolysate and

Table 3

Effect of Phentermine on Plaque Formation by Rickettsia conorii

Inoculum	Concentration of Phentermine (mg/ml)					
	None	0.1	0.2	0.4	0.8	1.6
<u>R. conorii</u>	42.5 \pm 5.2 ^a	40.0 \pm 4.8	30.3 \pm 6.0	16.3 \pm 2.5	14.0 \pm 2.2	Toxic
None	0	-	-	-	-	Toxic

a - Number of plaques/flask (mean \pm standard deviation)

Table 4

Effect of Indomethacin on Plaque Formation by Rickettsia conorii

Conc. Indomethacin	<u>R. conorii</u>		None	
	Day 6	Day 7	Day 6	Day 7
None	13.5 \pm 9.0 ^a	15.3 \pm 8.0	0	0
10 ⁻⁶ M	12.3 \pm 9.6 ^b	13.5 \pm 3.1	-	-
10 ⁻⁵ M	0 \pm 0	0 \pm 0	-	-
10 ⁻⁴ M	0 \pm 0	0 \pm 0	-	-
10 ⁻³ M	Toxic ^d	Toxic	Toxic	Toxic

a - Number of plaques/flask (mean \pm standard deviation)b - Plaques smaller than untreated R. conorii plaques

c - Partial destruction of monolayer by toxicity of indomethacin; no plaques in the intact portions of monolayer

d - Indomethacin toxic to monolayer at this concentration

Table 5

Effect of Phentermine Pretreatment of Rickettsia conorii on Plaque Formation

Inoculum (Phentermine Concentration)		Plaques ^a
<u>R. conorii</u>	(none)	TNTC
<u>R. conorii</u> + phentermine	(20 mg/ml)	TNTC
<u>R. conorii</u> + phentermine	(40 mg/ml)	TNTC
<u>R. conorii</u> + phentermine	(80 mg/ml)	15.8 \pm 4.3
<u>R. conorii</u> + phentermine	(320 mg/ml)	0 \pm 0

a - Number of plaques/flask \pm standard deviation

Table 6

Effect of Phentermine Pretreatment of Vero Cells on Subsequent Plaque
Formation by Rickettsia conorii

Conc. of Phentermine in Pretreatment	Plaques ^a
None	20.8 [±] 11.5
1 mg/ml	38.8 [±] 2.5
4 mg/ml	64.0 [±] 14.1
10 mg/ml	_b

a - Number of plaques/flask [±] standard deviation

b - Partial destruction of monolayer by toxicity of phentermine; estimated
adjusted mean of plaque counts, 86.5

Table 7

Effect of BABIM on Plaque Formation by Rickettsia conorii

Inoculum	Treatment (conc.)	Plaques
<u>R. conorii</u>	None	21.8 [±] 18.1
<u>R. conorii</u>	BABIM (10 ⁻⁴ M)	21.8 [±] 19.1
<u>R. conorii</u>	BABIM (10 ⁻⁵ M)	0 [±] 0
<u>R. conorii</u>	BABIM (10 ⁻⁶ M)	0 [±] 0
None	None	0 [±] 0
None	BABIM (10 ⁻⁴ M)	0 [±] 0
None	BABIM (10 ⁻⁵ M)	0 [±] 0
None	BABIM (10 ⁻⁶ M)	0 [±] 0

hemolysate fractions from the glucose-6- phosphate dehydrogenase deficient cells on R. conorii plaque formation, the effects of cholesterol receptor-binding compounds such as digitonin and amphotericin B on R. conorii plaque formation, and the effect of temperature on R. conorii plaque formation.

Conclusions:

My clinical observations in Sicily convince me that boutonuse fever is a potential military problem. The relative ease in finding bona fide cases and the high rates of seropositivity among the rural population indicate that circulation of spotted fever group rickettsiae is very active. Aside from lower mortality rates, the boutonuse fever problem appears analogous to scrub typhus in the Assam-Burma region during World War II.

We are in the process of defining the spectrum of illness associated with R. conorii. Our documentation of a patient who had a rickettsia-containing tache noire, but neither fever nor rash supplements the known range of disease. Our colleagues in Italy have shown that there are many persons who have no recollection of a boutonuse fever-like illness but do have antibodies reactive with R. conorii. We have also studied the pathology and distribution of R. conorii in two fatal cases of R. conorii infection in South Africa.

The pathologic lesions and pathophysiology of fatal RMSF and South African tick bite fever have both similarities and distinct differences. In general, the pattern of mononuclear leukocyte-rich infiltration of the infected blood vessel wall and perivascular space is the basic lesion in most affected organs in both RMSF and these two fatal cases of R. conorii infection. The lesions in the central nervous system from both diseases resemble the classic Frankel's nodules of typhus fever with cells compatible with macrophages and small and large lymphocytes permeating the perivascular neuropil. A mild leptomeningitis is observed in both rickettsioses. The cerebrospinal fluid in patient 1 reflected the rickettsial meningoencephalitis with mild pleocytosis and elevated protein concentration. Other analogous lesions include multifocal perivascular interstitial nephritis, focal interstitial myocarditis, dermal and subcutaneous vasculitis in the skin of the rash, eschar, and peripheral gangrene, and vasculitis in the pancreatic interlobular septa and periadrenal adipose tissue. Lesions which were observed in fatal R. conorii infection and are not characteristic of RMSF are necrotizing glomerular arteriolitis and multifocal necrosis of single hepatocytes. Although hepatocellular necrosis other than centrilobular necrosis was present in 5 of 9 fatal cases of RMSF in one series and in 7 of 16 cases in another series, the major hepatic lesions of RMSF, portal vasculitis and triaditis, were not observed in these two patients. The other major difference in pathology is the presence of interstitial pneumonia in RMSF and its absence in South African tick bite fever.

The distribution of lesions correlates with the locations of rickettsiae in each disease. Thus, the pulmonary capillaries and other small pulmonary blood vessels are infected with numerous R. rickettsii in RMSF, but there were few R. conorii infecting the pulmonary microcirculation in these two cases. Conversely, the glomerular arterioles in South African tick bite fever were the sites of intense R. conorii infection and necrotizing vasculitis but are not a target in RMSF. The locations of R. conorii and lesions correlated well in brain, meninges, liver, kidney, heart, spleen, skin, pancreas, and periadrenal arteries. These general

observations were confirmed in specific foci where R. conorii were observed in serial sections of vascular injury. Thus, these data support the theory of direct rickettsial injury of the parasitized cells proposed by Wolbach and supported by recent in vitro experiments.

A discrepancy in the correspondence of the injured cell and parasitized cell is noted in the liver. This description of R. conorii infection of sinusoidal lining cells is the first report of direct rickettsial infection of the liver in this disease; nevertheless, necrosis occurs in the hepatocyte adjacent to the infected sinusoidal lining cell. The mechanism by which this injury to the hepatocyte is mediated is not apparent. Previous investigations of lesions in hepatic biopsies have demonstrated accumulations of leukocytes in foci of hepatocellular necrosis. We have been unable to demonstrate intact R. conorii in any of these inflammatory lesions in humans. Experimental infections of mice with R. conorii have suggested that immune mechanisms clear the rickettsiae efficiently from the foci of the hepatocellular necrosis and inflammation of mice with intact T-lymphocytes while these lesions in T-cell deficient mice contain numerous rickettsiae. Moreover, those experiments and the absence of lymphocytes and macrophages in the foci of hepatocellular necrosis at the stage of development present in these cases would make cell mediated immunopathologic mechanisms seem unlikely.

The other lesions of particular interest are the amputated fingers. In addition to the expected observations of necrosis and wound repair, there was a zone of severely ischemic tissue in which a few viable cells persisted and organisms of R. conorii were identified. The observation of persistent rickettsiae in her tissues 36 days after the beginning of treatment with tetracycline is remarkable. Identical findings were present in amputated leg specimens from a patient in North Carolina with Rocky Mountain spotted fever who had been treated for three weeks with chloramphenicol. In both cases the spotted fever group rickettsiae were seen only in the ischemic partially viable zone where delivery of effective antirickettsial drug concentrations and host defenses such as T-lymphocytes, macrophages, and interferon was probably inadequate. This phenomenon may be related to the well known capability of spotted fever group rickettsiae to continue to proliferate in the yolk sac of hen's eggs for 48-72 hours after the death of the embryo. Proof that these morphologically intact organisms are truly viable will require isolation of rickettsiae from similar amputation specimens.

This documentation of the pathogenic potential of R. conorii in its most severe form indicates that further investigations of the renal, hepatic, neurologic, and cutaneous pathophysiology of this disease and of the pathogenic mechanisms of R. conorii should be pursued.

Our studies of the tache noire have shown that moderate-to-severe injury to numerous dermal blood vessels is the basis for the tick-bite site lesion. On the other hand, thrombosis was not as important as in the two previously investigated eschars in patients with RMSF. Among the 16 biopsies

of taches noires in which blood vessels could be identified for evaluation; there were no thrombi in 9, mild thrombosis in 5, and moderate or severe thrombosis in only 2. In contrast, edema was observed in all 16 cases in which it could be evaluated. By light microscopy, the predominant immune and inflammatory cell types in the vascular loci were small lymphocytes and large mononuclear cells. Polymorphonuclear leukocytes and eosinophils were generally inconspicuous. This observation will be further tested by ultrastructural examination; however, it supports the concept of the importance of cell mediated immunity possibly including local interferon production by T-lymphocytes.

Furthermore, this report documents the utility of skin biopsy immunofluorescence demonstration of R. conorii in the diagnosis of boutonuse fever. Spotted fever group rickettsiae were demonstrated in taches noires and the rash and could be identified by immunofluorescence on either frozen sections or deparaffinized, trypsin digested sections that had been fixed in formalin and embedded in paraffin previously. This technique should be made available on a reference basis to military health providers. The specific etiologic diagnosis of a few cases in an area sounds the alert for increased awareness of the disease and possible preventive measures directed against tick transmission.

Our human studies have also confirmed that boutonuse fever affects the liver. This hepatic injury is important to recognize in understanding the visceral involvement of this illness and as an in vivo model for the pathogenesis of R. conorii injury to tissues. In experimental mice, we have documented the importance of T-lymphocytes rather than B-lymphocytes reactive with carbohydrate antigens in immunity to R. conorii. Moreover, there was no apparent contribution of immunopathologic mechanisms to tissue injury in vivo.

Parabiotic chamber experimentation showed no diffusible exotoxin or soluble rickettsial enzyme that could injure cells in the identical milieu excluding the rickettsiae. On the other hand, two enzyme systems appear to play an important role in cell injury by R. conorii. Two inhibitors of phospholipase A2 were able to block plaque formation by R. conorii. Since pretreatment of the cells with one of the inhibitors (phentermine) resulted in an increase in quantity of plaques, it seems unlikely that the phospholipase is of host cell origin. In contrast, pretreatment of R. conorii with phentermine inhibited plaque formation. This plaque inhibition may have been due to killing of rickettsiae by phentermine or inhibition of a rickettsial phospholipase.

Moreover, there is evidence that BABIM, an inhibitor of trypsin-like proteases, is highly effective in blocking plaque formation by R. conorii. Thus a trypsin-like protease appears to be important in the physiology of and/or cell injury by R. conorii.

Recommendations:

1. Offer skin biopsy immunofluorescent demonstration of R. conorii in tache noire as a reference military laboratory diagnostic test for boutonneuse fever.
2. Study the rickettsial hepatitis of boutonneuse fever and experimental R. conorii infection as models for visceral tissue injury by R. conorii.
3. Initiate a search for nonpathogenic R. conorii or R. conorii-like isolates in ticks in Sicily.
4. Develop a more rapid, quantifiable assay system for growth of and cell injury by R. conorii for screening amidine type trypsin-like protease inhibitors as potential prophylactic or adjunctive therapeutic agents.
5. Screen amidine type protease inhibitors for inhibition of cell injury by R. conorii.
6. Continue studies of taches noires for role of thrombosis in cell injury and specific identification of local immune cell types.
7. Continue in vitro study of pathogenic mechanisms of cell injury by R. conorii.

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